Iranian Journal of Veterinary Science and Technology
Vol. 4, No. 1, 2012, 29-36

IJVST

Isolation and identification of *Brachyspira pilosicoli* from laying hens flocks, using conventional culture and molecular methods in Mashhad, Iran

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Received: July 13, 2011 Accepted: December 28, 2011

Abstract

Avian intestinal spirochaetosis (AIS) is an intestinal infection caused by anaerobic spirochaetes of the genus *Brachyspira*, including *B. pilosocoli*. The purpose of this study was isolation and identification of *B. pilosocoli* from laying hen flocks, located in Mashhad suburb, Khorasan Razavi province, Iran, and investigating the frequency of the infection. One hundred and eighty cloacal swab samples from 18 randomly selected flocks (10 samples /flock) were cultured anaerobically on selective agar and confirmed as intestinal spirochaete by its spirichaetal form using phase contrast microscopy. Then, the samples were subjected to PCR amplification followed by DNA sequencing. A total of 24 samples from 8 flocks were selected as suspected cases by culture and phase contrast microscopy. Upon PCR amplification by specific primers, only 9 cultures belonged to 3 flocks appeared to be *B. pilosicoli*. Sequence analysis of the amplicons confirmed the identity of all isolated ones. Based on the results obtained, it was concluded that *B. pilosicoli* might be strongly involved in AIS among laying hen flocks of this geographical region. The results could also be considered as an indicator for large scale investigation into the true prevalence of the infection. This study is the first report of infection in laying hens flocks of Iran.

Keywords: *Brachyspira pilosicoli*, laying hens, culture, PCR

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Introduction

The intestinal spirochaetes are all categorized in the genus *Brachyspira* (Mikosza and Hampson, 2001). To date, fifteen species of Brachyspira spp. have been described, including *B. hyodysenteriae*, *B. intermedia*, *B. murdochii*, *B. innocens*, *B. pilosicoli*, *B. alvinipulli*, *B. aalborgi*, *B. canis*, *B. corvi*, *B. ibaraki*, *B. rattus*, *B. muridarum*, *B. muris*, *B. pulli* and *B. suanatina* (The NCBI taxonomy databse: http://www.ncbi.nlm.nih.gov/taxonomy). The significance and consequences of Brachyspira infections in pigs (Hopwood *et al*., 2002) have extensively been described worldwide for decades, Brachyspira spp. have been isolated from pigs, birds, dogs, humans, non-human primates, guinea pigs, opossums and wild rodents (Hampson and Duhamel, 2006).

Avian intestinal spirochaetosis (AIS) is a condition arising from colonization of the caeca and colons of birds with anaerobic intestinal spirochaetes of the genus *Brachyspira* (formerly *Serpulina*) (Swayne, 1997; Stephens and Hampson, 2001). The condition occurs in commercial layers, layer and broiler breeders. The infection has been associated with a variety of production problems, including diarrhea, wet litter, faecal staining of eggshells, pasty vents, increased faecal fat content, delayed onset of egg laying, reduced egg weights, reduced growth rates, increased food consumption, poor digestion of food and increased number of weak chicks, with slower growth and poor food digestion (Davelaar *et al*., 1986; Griffiths *et al*., 1987; Dwars *et al*., 1990, 1992a, 1993; Swayne *et al*., 1992; Trampel *et al*., 1994; Smit *et al*., 1998).

Diagnosis of AIS is complicated by the fact that *Brachyspira* spp. are difficult to isolate, requiring at least 3 to 5 days of incubation using specialized selective media and anaerobic growth conditions.To date, three species of the genus have been identified as potential pathogens of chickens (McLaren *et al*., 1997), including *Brachyspira pilosicoli* (Trampel *et al*., 1994; Stephens and Hampson, 1999), *Brachyspira intermedia* (Griffiths *et al*., 1987; Dwars *et al*., 1992a,b, 1993; Stanton *et al*., 1997; Hampson and McLaren, 1999), and *Brachyspira alvinipulli* (Swayne *et al*., 1992, 1995; Stanton *et al*., 1998). In recent years, researches on the intestinal spirochaetes have been focused on classification, diagnostics, epidemiology and pathogenesis. Substantial achievements have been made due to new technologies based on molecular biology, eg DNA–DNA reassociation, polymerase chain reaction (PCR), DNA sequencing, pulsed-field gel electrophoresis (PFGE) and recombinant DNA-technology. Based on PCR method combined with traditional culture technique and biochemical tests, a number of new spirochaetal species have been identified (Atyeo *et al*, 1998., Mikosza *et al*, 2001). Improved knowledge on the genetic organization, the ultrastructure and virulence factors have been gained through the use of the novel techniques. The purpose of this study was to investigate the presence of the anaerobic intestinal *Brachyspira* spp. amongst laying hens flocks located in Mashhad suburb of Iran using PCR method.

Materials and Methods

Sampling

180 cloacal swab samples were collected from 18 randomly-selected flocks (10 samples from each flock), in Mashhad suburb of Khorasan Razavi province, Iran. The age of flocks ranged from 10 to 20 months.

Culture of faecal samples

The rectal swabs were plated onto Trypticase Soy agar supplemented with 5% defibrinated bovine blood, 400 µg mL^{-1}spectinomycin, 25µg mL^{-1} of vancomycin and 25 µg mL^{-1} of colistin. Plates were incubated at 37°C in anaerobic jars in an atmosphere of 94% N2 and 6% CO2 for 10 days. Spirochaete growth was indicated by a zone of weak β-haemolysis, surrounding a low flat haze of bacterial growth. The presence of spirochaetes was examined by direct examination of bacterial growth suspension in phosphate buffered saline (PBS; pH 7.2) under a

phase contrast microscopy at 400× magnification. These colonies were selected for more analysis.

DNA extraction and PCR amplification

Chromosomal DNA was extracted and purified from the suspected colonies on modified Trypticase Soy agar, using phenol-chloroform procedure (Simon *et al.,* 1996). The final pellet of DNA were resuspended in 100µL TE buffer and stored at -20 °C. The extracted DNA was used as a template for PCR amplification.A 439 base pair sequence of the 16S rRNA gene of *Brachyspira pilosicoli* was targeted for PCR amplification using the 16S/2pil primer pair (F-Acoli1- 5'-AGA GGA AAG TTT TTT CGC TTC-3' and R-647 16S- 5'- CCC CTA CAA TAT CCA AGA CT-3') was used in this study (Mikoza *et al* 2004). For PCR reaction, the amplification mixtures consisted of a 25 µL reaction mix of 2.5µL PCR buffer (10X), 0.5µL of dNTPs mix (10mM), 1µL of MgCl2 (50mM), 0.2µL DNA Polymerase(5U/µL), 12.5 pmol of each primer, 2µL DNA sample, and 16.3µL of DW. Cycling Amplification conditions involved a 5 min denaturing step at 94°C, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 51°C for 30 s, and a primer extension at 72°C for 30 s. After the last cycle the product incubated at 72°C for 5 min. The PCR products were subjected to electrophoresis in 1% (w/v) agar gels in 1×TAE buffer (40 mM Tris-acetate, 1 mM EDTA). The current voltage for electrophoresis is set at 60 Volts for 50 min. The bands were stained by emersion in 0.5 µg ml^{-1} ethidiumbromide for 30 min, and the gels were viewed and recorded by gel documentation apparatus.

Positive control

Due to the lack of positive control, the initial suspected isolated one was subjected to DNA extraction and PCR amplification followed by DNA sequencing of the 439bp product size.

Results

Sequence analysis of the 439bp PCR confirmed the identity of the isolate (98% confirmation of the identity with most *B. pilosicoli* isolates deposited in gene bank database).This isolate was subsequently used as a positive control throughout the study. From 180 rectal swab samples collected, 24 isolates belonging to 8 flocks were obtained, upon immediate swabbing and selective plating.The morphology of the isolates was confirmed by phase contrast microscopy. In PCR assay, employing specific primers of *B. pilosicoli*, a 439 base pair product of the 16S rRNA were only amplified in9samples. These positive samples represented 5% of total 180 rectal swab samples examined (Figure 1). As the positive samples belonged to three flocks, it was concluded that 16.7% of flocks investigated were infected with B. pilosicoli.

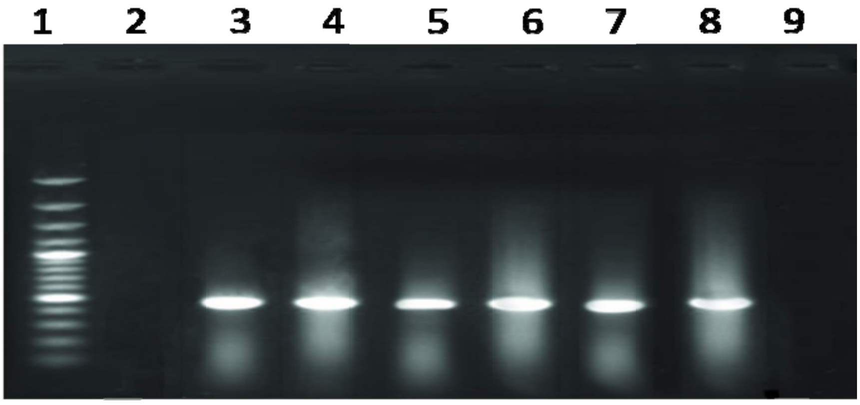


Figure 1. Detection of *B. pilosiloli* in faecal bacterial isolates by PCR assay, amplifying 439bp segment of 16SrRNA gene: Lane (1) 100 bp plus marker, lane (2 and 9) negative control, lane (8) positive control, lanes (3, 4, 5, 6 and 7) samples.

Discussion

Intestinal infection of laying birds with *B. Pilosicoli* can cause a drop of 5% in egg production with no impact on mortality (Swayne, 2003). However, if the condition remains undiagnosed and untreated, and lasts for the full length of the laying period, the effect can reach a 6% reduction in egg production and an increase of mortality by 8.84%.

According to the results obtained, 16.7% of laying hens flocks were infected with *B. pilosicoli.* This rate of infection is somehow similar to the prevalence of the infection in commercial layer flocks in UK (14%) (Thomson *et al.,* 2007), but the flocks had a history of 'wet droppings' if their samples were collected randomly, as performed in our study, it was possible that the reported prevalence rate was much lower.

In contrast, the rate of infection in our pilot study was higher than the rate in Netherlands, in which only 4.4% of flocks with no history of enteric signs were reported to be infected (Dwars *et al*., 1989).

The injudicious use of antibiotics in commercial layers in the region, may promote the prevalence of the infection through suppressing normal GI microflora and leading to reduce colonization resistance.This phenomena, may accelerate the spirochaetal colonization, as reported elsewhere (Jamshidi and Hampson, 2002).

In our study, the method of isolation was based on the streaking of cloacal swabs on selective medium, in farm environment and immediate transfer of the inoculated plates toanaerobic jars. We did not use pre-treatment step, a useful practice for enhancing the survival and selection of the spirochaetes (Calderaro *et al*., 2005). Instead of immediate culture and rapid transfer of the inoculated transfer media to laboratory, we employed inoculation of agar media in farm to prevent losing bacteria upon transportation to the laboratory. This practice, although it might not be perfect, but could reduce the chance of missing some positive samples. By the way, if the enrichment was conducted, the prevalence of infection might be more than that is reported.

Although PCR assays for *Brachyspira* species have been conducted on DNA samples extracted from infected human and pig faeces (Mikoza *et al*., 2001, Choi *et al*., 2002, La *et al*., 2003, La *et al*., 2006), direct PCR on faecal samples was not employed in our study. According to the literature, so far direct PCR on chicken faecal samples has not been successful. The reason is likely to be associated with the low pH of chicken faeces, and the presence of uric acid and other PCR inhibitors. By the way, washing processes may be effective at removing potential PCR inhibitors from chicken faeces (Nyree *et al* 2006).

There is no doubt, the sensitivity of detection of *B. pilosicoli* byfaecal culture is not particularly high [>5 x 10^4 cells $(gfaeces)^{-1}$] (Atyeo *et al* 1998). Therefore it is possible thatinvestigators mayencounter some false negative flocks in their studies based on bacterial culture. (Mikosza *et al.,* 2001)

*B. pilosicoli*as a potential zoonotic bacteriacan infect across species boundaries (Jamshidi and Hampson, 2003). Therefore the rate of infection in laying hen flock (16.7%) in Mashhad suburb may put the population at risk of the zoonotic infection. Determination of true prevalence of infection in rural and urban regions of the country, may put enough shed in the level of risk of human population.

The distribution of human *B. pilosicoli* infections shows a remarkable polarization, with the infection being common (10-30%) in developing countries, but rare in the general population in developed countries. In Oman, a prevalence of 15% was found amongst Persian Gulf (Barrett, 1990) and in Papua New Guinea the prevalence has been reported about 22.8% (Trott *et al*., 1997).

In this study no attempt was done to characterize strain of the isolated *B. pilosicoli*, as it needs pulsed field gel electrophoresis (PFGE) assay. The results are also an excellent indicator for sample size

determination for a large scale investigation of true prevalence of the infection in the respected geographical region. This study is the first report of *B. pilosicoli* infection in laying hens flocks of Iran.

Acknowledgments

Financial support by the Faculty of Veterinary Medicine, Ferdowsi University of Mashhad is greatly appreciated.

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Iranian Journal of Veterinary Science and Technology
Vol. 4, No. 1, 2012, 29-36

IJVST

جداسازی و شناسایی عامل اسپیروکتوز روده‌ای انسان (برکیسپایرا پیلوسی کولای) از گله‌های طیور تخم گذار با استفاده از روش کشت مرسوم و روش مولکولی در شهرستان مشهد

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دریافت مقاله : ۱۳۹۰/۴/۲۲ *پذیرش نهایی: ۱۳۹۰/۱۰/۷*

چکیده

اسپیروکتوز روده‌ای طیور (AIS) یک عفونت روده‌ای است که عامل آن اسپیروکت‌های بیهوازی از جنس برکیسپایرا از جمله برکیسپایرا پیلوسی کولای می‌باشد. هدف از انجام این مطالعه جدا سازی و شناسائی باکتری برکیسپایرا پیلوسی کولای از گله‌های طیور تخمگذار در اطراف شهرستان مشهد و نیز تخمینی از فراوانی عفونت در گله‌ها بود. در این مطالعه تعداد ۱۸ گله طیور تخمگذار در اطراف شهرستان مشهد، بصورت تصادفی انتخاب گردید و از هر گله تعداد ۱۰ سواب رکتال نمونه برداری شد. سواب‌ها بصورت بیهوازی در محیط آگار انتخابی کشت داده شد، سپس کلنی‌های تیپیک با استفاده از میکرسکپ فاز کنتراست بر اساس شکل باکتری به عنوان اسپیروکت روده‌ای مورد شناسائی قرار گرفت. از نمونه‌های مشکوک تست PCR و سپس تعیین توالی DNA صورت گرفت. براساس کشت و مشاهده زیر میکروسکوپ فاز کنتراست تعداد ۲۴ نمونه مربوط به ۸ گله به عنوان نمونه‌های مشکوک و در تست PCR با استفاده از پرایمرهای اختصاصی تعداد ۹ نمونه کشت شده مربوط به ۳ گله به عنوان برکیسپایرا پیلوسی کولای مورد شناسائی قرار گرفت و آنالیز توالی قطعات تکثیر یافته هویت جدایه‌ها را مورد تأیید قرار داد. بر اساس نتایج بدست آمده مشخص گردید که گله‌های طیور تخمگذار در این منطقه جغرافیائی آلوده به این باکتری می‌باشند. این نتایج همچنین می‌تواند به عنوان یک پایه مناسب برای مطالعات گسترده تر جهت تعیین میزان شیوع عفونت مورد استفاده قرار گیرد. این مطالعه اولین گزارش عفونت به باکتری برکیسپایرا پیلوسی کولای در گله‌های طیور تخمگذار در ایران می‌باشد.

واژگان کلیدی: برکیسپایرا پیلوسی کولای، طیور تخم گذار، کشت، PCR